Imbalance of pH and Potassium Concentration as a Cause of Stuck Fermentations

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The effect of the ratio of potassium to hydrogen ions on fermentation rate and progression was evaluated in conditions mimicking grape juice. A molecular K^+/H^+ ratio of 25:1 was found to be the minimum required for the completion of fermentation. Decreases in potassium concentration resulted in the failure to metabolize available glucose and fructose causing a stuck fermentation. If potassium levels were too high relative to the pH, the decrease in pH brought about by potassium uptake lowered the medium pH to a level at which it became inhibitory to continued fermentation. Thus, the ratio of potassium to hydrogen ion concentration will lead to a problem in progression of the fermentation if it is either too low or too high. The ratio of potassium to hydrogen ion did not affect rate of growth or formation of maximal cell biomass, but it did slightly impact maintenance of cell viability, which could be a contributing factor to the arrest of fermentation. Fermentation arrest was not detectable until three days post-inoculation; however, supplementation with potassium at that or later times did not restore fermentation rate. Grape juice at pH 3.3 or below may be at risk of premature arrest of fermentation due to a deficiency or excess of potassium, depending upon the buffering capacity of the juice.

KEY WORDS: *Saccharomyces*, stuck fermentation, potassium, pH

Fungi take up potassium in exchange for hydrogen ions in order to balance cytoplasmic acidifying processes while maintaining ionic stasis [2,5,6,7]. In addition to playing a key role in the adjustment of cytoplasmic pH, potassium is also known to accelerate the rate of glucose consumption in the yeast *Saccharomyces* [2,13]. Glucose consumption rates are significantly faster in the presence of potassium than in its absence at low pH [9,12,13,14]. The increase in rate of glucose uptake is coincident with the uptake of external potassium and the release of protons from the cell [13,14]. It has been proposed that the decrease in medium pH is a consequence of excretion of protonated organic acids, a process stimulated by potassium uptake rather than due to the presence of a specific potassium/hydrogen ion antiport [13]. The uptake of potassium appears to be driven by the hydrogen ion and potassium concentrations of the medium and not by internal pH or cytoplasmic potassium concentration [9,12,13,14]. The pH optimum of fermentation decreases as the concentration of potassium increases [9], suggesting a complex interrelationship between these two ions.

The mechanism by which potassium stimulates glycolysis is largely unknown. However, the effect appears to be mediated at the level of transport of sugar into the cell [9,12,13,14]. Potassium leads to a rapid stimulation of glucose uptake that accounts for the overall increase in fermentation rate [9,12], but does not appear to stimulate the activity of downstream enzymes of glycolysis [9,12]. Only external potassium is stimulatory as the cytoplasmic concentration of potassium seems to have no effect on glucose consumption [11], again suggesting that the target of potassium activation is not a cytoplasmic factor, but one located at the cell surface. The recent discovery that mutant forms of glucose transporters can mediate uptake of potassium and restore growth to cells lacking potassium transporters [3], suggests that potassium and glucose uptake may be mediated by the same proteins. In fact, it has been proposed that uptake of these two substrates is directly coupled via a symport mechanism [3]. In this case glucose, which is always present in greater concentration outside of the cell, may drive uptake of potassium against its concentration gradient. However, glucose uptake is not dependent upon the presence of potassium. Thus, if symport occurs, it is not obligatorily coupled to the uptake of sugar.

The stimulation of glycolysis by external potassium was first demonstrated by Pulver and Verzar in 1940 [10], with the discovery that potassium was taken up during the active phase of fermentation and released post-fermentation [10]. Subsequent work has demonstrated that the stimulation of glycolysis persists after the uptake of potassium has ceased [9]. However, these earlier studies did not follow the progression of a fermentation, but merely measured maximal fermentation rate as a function of potassium concentration in the medium. Further, previous work was not conducted in media representative of grape juice. The glucose concentration of grape juice is significantly greater than that used in previous studies. Also, high ethanol concentrations can alter proton flux across the cell membrane, which was not considered in the earlier

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work. It is possible, therefore, that the potassium concentration best sustaining fermentation under enological conditions might differ from that allowing an initial high fermentation rate. The goal of this work was to investigate the relationship between pH and extracellular potassium under enological-like conditions. Further, the previous studies only examined the effect on glucose consumption over very short time periods. The observation that the potassium effect persists suggested that an imbalance of potassium and hydrogen ion concentrations might lead to sluggish or stuck fermentations. Therefore, the effect of potassium and hydrogen ion ratios was evaluated over the entire time course of fermentation.

Materials and Methods

Reagents and yeast strain: All chemicals were reagent grade unless other wise noted. Vitamin assay casamino acids and Bacto-peptone were from Difco (Detroit, MI). The yeast strain used was UCD522, also known as Montrachet. A commercial active dry yeast preparation of the strain was obtained from the Universal Foods Corporation (Milwaukee, WI).

Media: A modification of the "Triple M" medium of Ralph Kunkee [4] was used. The main modification was a reduction of the amount of tartaric acid, necessitated by the fact that at high levels of potassium, potassium will form a salt with tartrate and precipitate from the medium. As such, the buffering capacity of the medium may be somewhat less than a typical grape juice. The medium contained in grams/L: D-glucose (130); D-fructose (130); L-Tartaric acid (3.0); L-malic acid (3.0); citric acid (0.5); KH_2PO_4 (1.0); $CaCl_2 \cdot 2H_20$ (0.37); NaCl (0.1); $MgSO_4 \cdot 7H_20$ (1.025); Vitamin assay casamino acids (2.0); L-Arginine-HCl (0.8); L-proline (1.0); L-tryptophan (0.1). Ergosterol was added at 10 mg/L and Tween 80 at 1 mg/L. In addition, 1.0 mL/L each of a vitamin and trace elements mixture was added. One liter of the concentrated vitamin mixture contained: biotin (2 mg); calcium pantothentate (400 mg); folic acid (2 mg); myo-inositol (8000 mg); nicotinic acid (400 mg); *p*-aminobenzoic acid (200 mg); pyridoxine HCl (400 mg); riboflavin (200 mg) and thiamin HCl (400 mg). One liter of the concentrated trace elements mixture contained: boric acid (500 mg); copper sulfate (40 mg); potassium iodide (100 mg); ferric chloride (200 mg); manganese sulfate (400 mg) sodium molybdate (200 mg); and zinc sulfate (400 mg). Additional potassium was added as KCl or as K_2SO_4 where indicated. This synthetic medium is representative of a grape juice, but with all nutrients in excess. Control experiments indicated that fermentation rate was not limited by either the macro- or micronutrient content. The impact of altering the ratio of individual components can thus be evaluated in this medium. However, this medium does not contain the non-nutrient components of grape juice, such as the phenolic compounds.

Ammonium hydroxide was used for the adjustment of pH of the media, because other cationic species have been reported to interfere with the potassium effect on stimulation of fermentation rate [9]. Sodium hydroxide could not be used, as sodium is toxic to yeast cells. Control fermentations indicated that the differences in ammonium ion concentration due to differential addition of ammonium hydroxide did not impact fermentation rate.

Fermentation trials: An active dry yeast preparation of strain Montrachet was rehydrated according to manufacturer's instructions. The rehydrated cells were added at a concentration of 1×10^6 viable cells/mL to 300 mL of modified triple M medium containing only 1% glucose. After three days of growth at 20°C, a portion of the culture was transferred to 300 mL of the 26% modified triple M medium contained in a 500 mL flask, again at an initial concentration of 1×10^6 viable cells/mL. Fermentations were conducted at 20°C on a rotary shaker (140 rpm). Sugar consumption was monitored by measuring weight loss of evolved carbon dioxide. Between two to four replicate experiments were run for each condition. The replicates did not vary greater than 10%. Since replicate experiments were run, and not replicate samples, the data is presented for a single experiment only.

The pH was monitored over the course of the experiments to document the decrease in pH as a function of potassium concentration. pH values reported represent the average of three independent measurements. Replicate pH measurements varied by 0.03 units or less.

Total and viable cell count: Total cellular biomass was monitored in two ways, by determining total cell count using a Levy counting chamber (Hausser Scientific, Blue Bell, PA) and by absorbance and 580 nm using Spectronic 1201 (Milton Roy, Rochester, NY). Viable counts were determined in triplicate with YEPD (10% yeast extract, 20% Bacto-peptone, and 2% glucose) as the medium.

Potassium measurement: Potassium concentrations were measured either by atomic absorption or emission, depending upon the concentration. Samples were diluted 1:100 with 0.1% cesium in 0.01 *N* HCl, following removal of the cells by filtration through a 0.45-µm membrane filter. For atomic absorption, the standards containing 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L of potassium were used. For concentrations of more than 4 mg/L, analysis was by atomic emission. The atomic emission standards contained 5, 10, 15, 20 and 25 mg/L of potassium.

Residual sugar: Residual sugar was measured using the D-glucose/D-fructose Food Analysis Kit (Boehringer Mannheim, Germany). Residual sugar values are reported as the sum of glucose and fructose. Fermentations were considered to be stuck if greater than 0.25 g/L residual sugar (glucose + fructose) remained.

Results and Discussion

Potassium deficiency leads to a stuck fermentation: The effect of the concentration of potassium on

progression of fermentation of a juice-like synthetic medium was investigated. Potassium levels were varied from 300 to 2000 mg/L in a model juice fermentation medium at pH 3.3. All values yielded an identical maximal fermentation rate, but the 300 mg/L sample stuck (Fig. 1A) at a residual sugar concentration of approximately 4.4% (Table 1). The 500 mg potassium levels also displayed a high level of residual sugar (2.2%). Residual sugar concentrations were still high (approximately 1%) in the samples receiving high levels of potassium. This inhibitory effect of high potassium appears to be due to the decrease in medium pH associated with potassium uptake, as described below. Thus, the level of potassium relative to the hydrogen ion concentration required for completion of a juice fermentation is greater than that required to obtain a maximum rate. The rate of fermentation is not sustained if potassium is limiting or in excess. Previous studies determined that the minimum molecular ratio of potassium to hydrogen ions required for maximal stimulation of fermentation rate was 12:1 [12]. However, in our study, fermentation was arrested prematurely if the K^+ /H^+ ratio was less than 25-30:1. Since potassium can stimulate proton excretion from cells [9,12] and ethanol leads to a higher rate of passive proton flux [1], the requirement for high potassium may be a consequence of the higher ethanol levels late in fermentation.

Potassium requirement for completion of fermentation is dependent upon pH: Earlier studies indicated that the stimulation of fermentation rate by potassium is pH-dependent with higher potassium concentrations required at lower pH values [14]. Therefore, the effect of pH on the level of potassium needed to complete a fermentation was evaluated. At pH 3.0 (Fig. 1B), even 1000 mg/L was insufficient to allow completion of the fermentation. Residual sugar concentrations were higher at all potassium levels as compared to pH 3.3 (Table 1). In contrast, at pH 3.6 all potassium levels allowed fermentation to go to dryness (Fig. 1C), and residual sugar concentrations were low in all cases (Table 1). Thus, the potassium concentration needed for completion of a fermentation was dependent upon medium pH, and the lower the pH the higher the potassium requirement.

Table 1. Levels of total residual sugar at the end of fermentation at varying ratios of potassium to hydrogen ion concentration.

	Residual sugar concentration (g/L)*		
Potassium (mg/L)	pH 3.0	pH 3.3	pH 3.6
300	76.3	44.3	0.19
500	56.9	22.8	0.13
700	43.5	15.1	0.13
1000	37.5	12.5	0.13
1500	26.6	11.1	0.17
2000	25.0	12.2	0.21

*Expressed as total of glucose and fructose at 320 hours post-inoculation. Values are from the fermentations depicted in Figure 1. Replicate fermentations differed in residual sugar content less than 10%.

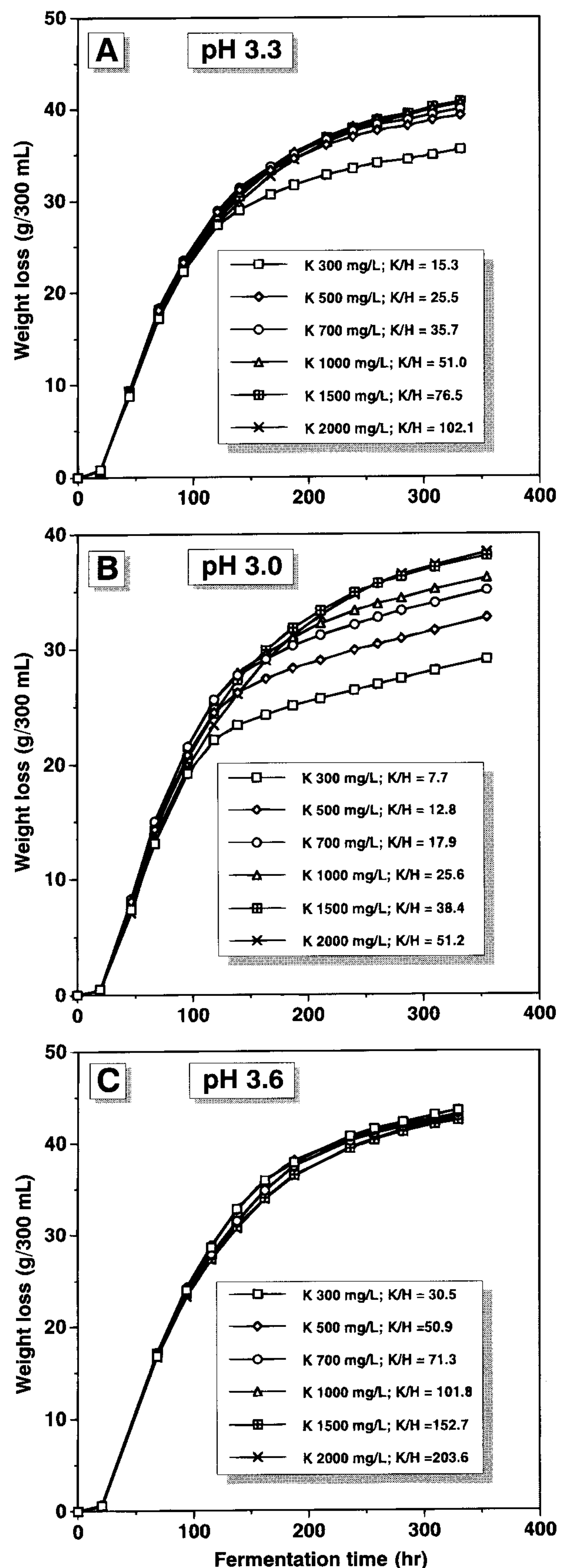

Fig. 1. Effect of potassium level on fermentation progression at three pH values. pH 3.3 (Panel A); pH 3.0 (Panel B); pH 3.6 (Panel C). Potassium concentrations are indicated in the side panel.

In the experiments described above, potassium was always added in the form of KCl . Although the observed stimulation was far more likely the effect of potassium than of chloride, the possibility that chloride was stimulatory could not be eliminated. To determine if the presence of the chloride ion might influence the results, the experiment at pH 3.0 was repeated using potassium sulfate as the source of potassium. The same stimulation was obtained when potassium was added as the sulfate salt (Fig. 2), which confirms that the effect was due to the potassium.

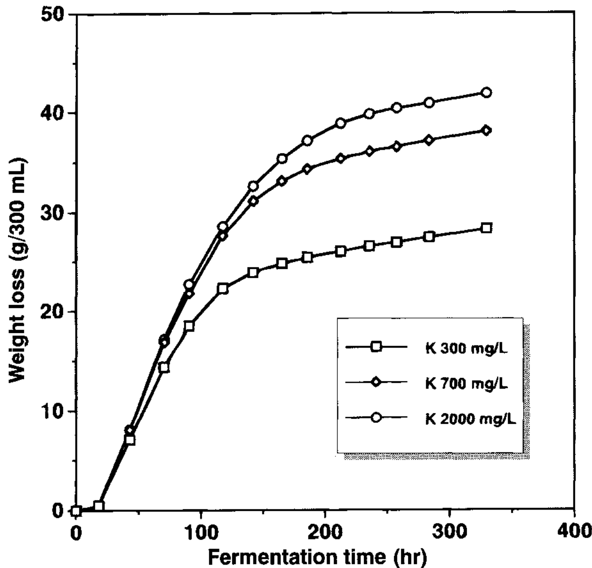

Fig. 2. Effect of potassium supplementation as K_2SO_4 at pH 3.0. The concentration of potassium is indicated in the side panel.

For grapes harvested in California, a range of 560 to 2785 mg/L of potassium with an average of 1846 mg/L has been reported [8]. At pH 3.3 and below, the extremes of potassium concentration observed could lead to arrest of fermentation, based upon the results obtained with the grape juice synthetic medium. UCD522 was chosen for this study because it has a high fermentative capacity and the impact of a deficiency of potassium might be more or less severe in other strains.

Nitrogen and sulfate supplementation do not alter response to low potassium: The presence of other monovalent cations in the medium has been shown to alter the potassium effect on fermentation [9,12]. This poses a problem with respect to which chemical species to use for adjustment of the pH of the synthetic medium. Since ammonium ion had only a slight effect on potassium stimulation of fermentation [9,12], ammonium hydroxide was used to adjust medium pH. However, the use of ammonium hydroxide leads to addition of a nitrogen source. Triple M medium is not limiting for nitrogen, but the possibility that the presence of additional ammonium was stimulatory could not be eliminated. To test the possibility of an interference by ammonium ion, fermentations were conducted with and without ammonium sulfate supplementation of the medium (Fig. 3). Supplementation

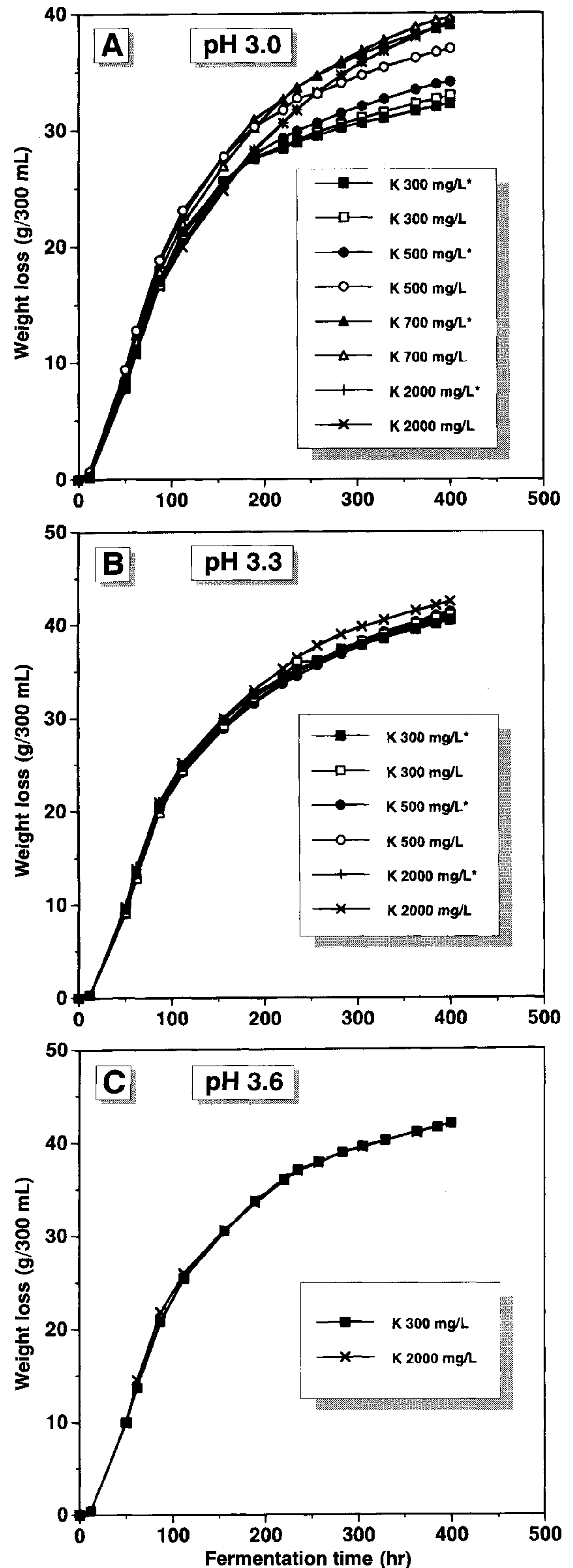

Fig. 3. Effect of ammonium sulfate addition on the potassium-mediated stimulation of fermentation at pH 3.0. Samples marked with an asterisk received ammonium supplementation up to 750 mg/L, which was equivalent to the amount present in adjustment of pH to 3.6.

was not able to prevent the fermentation from sticking and did not affect fermentation rate at all. Therefore, the cells were not limiting for nutrients, and differences in ammonium or sulfate concentration had no impact on fermentation rate or progression.

Potassium level affects the degree of acidification of the medium by yeast. The exchange of potassium for hydrogen ions leads to a decrease in the pH of the medium [13]. To determine the extent to which the pH is decreased as a function of potassium concentration, the pH of the medium was monitored during the course of these fermentations. The level of potassium had a dramatic impact on the final pH of the medium (Fig. 4). The drop in pH, especially at the low initial pH values was significant. The drop in pH at high potassium concentrations lowers the medium pH to a level that itself becomes inhibitory to fermentation. Thus, the high residual sugar concentration observed in the cases of high potassium is likely due to the arrest of fermentation caused by the decrease in pH. Therefore, if the potassium level is too high relative to the initial pH, the fermentation may also arrest prematurely due to the development of an inhibitory pH. The magnitude of this effect is also dependent upon the buffering capacity of the medium or juice.

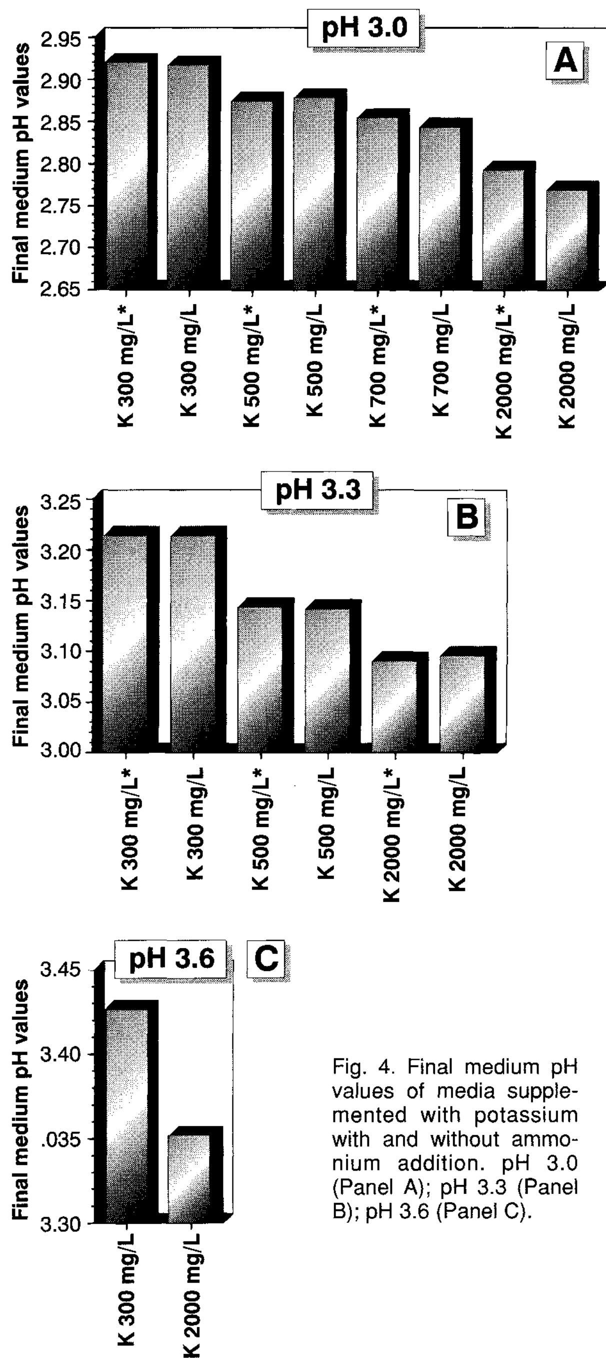

Fig. 4. Final medium pH values of media supplemented with potassium with and without ammonium addition. pH 3.0 (Panel A); pH 3.3 (Panel B); pH 3.6 (Panel C).

Time of addition of potassium to potassium-limited fermentations affects ability to correct the deficiency: The effect of time of addition of potassium to a potassium-limited fermentation was investigated. For this study, medium at pH 3.0 supplemented to 300 mg/L of potassium was used. At specific time points during the course of the fermentation, additional potassium, 1700 mg/L, was added (Fig. 5). In previous experiments at pH 3.0, fermentation rate at low potassium concentrations was identical to that at the higher concentrations for the first three days of the fermentation, becoming sluggish thereafter and stopping at approximately day 5 or 6. Potassium was added to the fermentation at 72, 96, 120, and 168 hours as well as at the start of the fermentation.

Potassium supplementation at 72 and 96 hours stimulated fermentation and resulted in a more complete fermentation, but was not as effective as supplementation of the starting juice. Supplementation at later time points did not stimulate fermentation. Thus, addition of potassium to fermentations that have already arrested is not effective in stimulation of the fermentation. Further, addition at 72 hours, a time point prior to the detection of a reduction in fermentation rate, is only partially effective. This suggests that

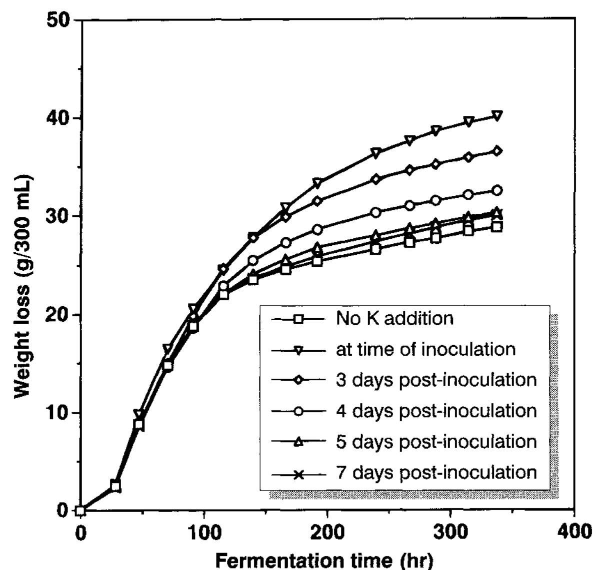

Fig. 5. Effect of timing of addition of potassium on fermentation rate of potassium deficient fermentations.

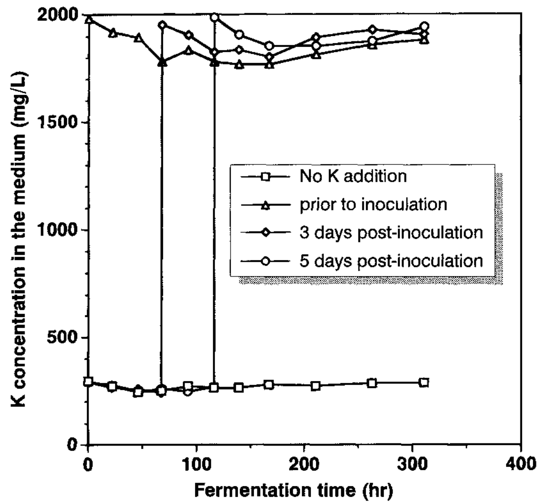

Fig. 6. Medium potassium concentrations during fermentation of potassium deficient and potassium supplemented cultures. The medium pH as 3.0.

the fermentation is not stuck due to a simple limitation for potassium but that the low potassium levels have altered the physiological properties of the cells. This is in excellent agreement with the early observations of a persistence in the effect of potassium on fermentation rate following cessation of potassium uptake.

The potassium concentration in the medium was monitored over the course of the fermentations in order to investigate the relationship between potassium uptake by the cells and the effectiveness of potassium supplementation late in the fermentation. For all of the conditions, potassium was taken up into the cells during the early stage of fermentation, and later, during the final stages was released into the medium (Fig. 6). This is quite consistent with the original observation of Pulver and Verzar [10]. Maximum potassium uptake was approximately 50 mg/L without addition of potassium and 210 mg/L when potassium was present at the start of fermentation, representing 16% to 10.5%, re-

Fig. 7. Total cell count, biomass yield and viable cell counts during fermentation at pH 3.0 with limiting potassium concentration, and following supplementation with potassium (1700 mg/L) at different times during the course of the fermentation. Panel A: Fermentation profile; Panel B: Total cell count; Panel C: Absorbance; Panel D: Viable cell count. Symbols are the same as those for Figure 6.

spectively, of the potassium available. This again confirms that it is the external ratio of potassium to hydrogen ion concentration that is crucial for the stimulation of fermentation. If supplementation occurred at three days post-inoculation, potassium uptake was the same as that observed in the fermentation that received potassium at the start. Potassium addition at five days also resulted in uptake of potassium, of approximately 175 mg/L. Thus, addition of the potassium does lead to increased uptake of the ion, but does not restore fermentation rate. It appears that the amount of ion taken up is poorly correlated with fermentation progression, in agreement with the observations that external concentration of potassium is most critical [9,12,13,14].

Effect of potassium limitation on cell growth and viability: Potassium deficiency may impact fermentation rate in one of two ways, by reducing the fermentation capacity of the individual cells or by reducing the fermentation capacity of the culture through the loss of cell viability. Therefore, total cell count and viable cell count was monitored during the course of potassium limited fermentations (Fig. 7A). There were no consistent differences in cell growth regardless of the initial potassium concentration (Fig. 7B, C). Further, loss of cell viability and decrease in fermentation rate were somewhat correlated (Fig. 7A, D). Cell viability was greatest when supplementation of potassium occurred early, at the start of fermentation or after three days of fermentation, versus no supplementation or supplementation occurring after five days of fermentation. Thus, potassium deficiency impacts cell viability as well as fermentation rate but does not appear to affect growth rate or development of maximal biomass.

Analysis of the potassium levels and pH of California juices suggests that in most cases sufficient potassium is present. In some cases, however, an excess of potassium appears to be present which may limit low pH fermentations, depending upon the overall buffering capacity of the juice. However, much of this compositional work was based upon fruit from vines on the A×R1 rootstock. Vine potassium status varies as a function of rootstock and some of the other rootstocks now in common use result in less uptake of potassium. Vintners working with low pH juices (pH 3.3 or below) should be aware of the potassium level of the juice and the potential for fermentation arrest if the level is either too high or too low.

Conclusions

An imbalance of potassium and hydrogen ion concentrations was shown to lead to a stuck fermentation in a synthetic grape juice medium. Fermentation arrested if the potassium concentration were too low or too high, the latter case likely due to the decrease in pH that occurs with high potassium levels. Potassium additions were able to stimulate the fermentation of a potassium-deficient juice only if added early in the fermentation, prior to the observed decrease in rate of fermentation. Potassium deficiency decreased cell viability late in fermentation suggesting that the potassium is required as a survival factor.

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